



Yeastern Biotech Co., Ltd



YLEX
EXPRESSION KIT

About the Kit

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Address: 6F-3, 23 Lane 169, Kang Ning St., Shijr, Taipei, 22180 Taiwan.
Tel: +886-2-2695-3922 Fax: +886-2-2695-3979
Email: yeastern@yeastern.com Website: www.yeastern.com

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Please complete and return the enclosed Product User Registration Agreement for each YLEX Expression Kit that you purchase. This will serve as a record of your purchase and registration and will allow Yeastern Biotech to provide you with technical support and manual updates. It will also allow Yeastern Biotech to update you on future developments of and improvements to the YLEX Expression Kit. The agreement outlined above becomes effective upon our receipt of your User Registration Card or 10 days following the sale of the YLEX Expression Kit to you. A use of the kit at any time results in immediate obligation to the terms and conditions stated in this Agreement.

Technical Services

Yeastern Biotech provides Technical Services to all of our registered YLEX Expression Kit users. Please contact us if you need assistance with the YLEX Expression Kit.

Kit Components

Yeast Strain: Po1g

The strain Po1g of *Yarrowia lipolytica* is a derivative of the wild-type strain W29 (ATCC 20460) by a series of genetic modifications. Briefly, the original *URA3* gene in the W29 strain was disrupted with the *SUC2* gene from *Saccharomyces cerevisiae*, followed by the introduction of a deletion in the *LEU2* gene. Furthermore, the deletion of the *XPR2* and *AXP* genes ensures that Po1g is unable to produce any extracellular protease. In order to allow easy integration of pBR-based expression/secretion vectors, a pBR322 docking platform was integrated at the *URA3* locus. (Madzak *et al.*, 2000)

Strain	Genotype	Phenotype
Po1g	MatA, <i>leu2-270, ura3-302::URA3, xpr2-332, axp-2</i>	Leu ⁻ , ΔAEP, ΔAXP, Suc ⁺ , pBR platform

Vectors (Madzak *et al.*, 2000) :

	Name	Amount
Expression	pYLEX1 (noted as pINA1269 in Madzak <i>et al.</i> , 2000)	5 µg
Secretion	pYLSC1 (noted as pINA1296 in Madzak <i>et al.</i> , 2000)	5 µg

Primers: (for DNA sequencing purpose) :

Type	Sequence	Amount
6560 F	5'-GAT CCG GCA TGC ACT GAT C-3'	250 µl
6904 R	5'-AAC ACC GGT GTT GGA CTC AG-3'	250 µl

YLOS Transformation Kit (Chen *et al.*, 1997) :

Component	Description	Storage Temp	Quantity
YLOS Buffer	A mixture of cations and polyethylene glycol	Room Temp	12 ml (1.5 ml x 8)
Carrier DNA	A mixture of single strand DNA for enhancing the transformation efficiency	Dispense into 50 µl aliquots, stored at -20°C	0.3 ml
DTT powder	A reducing agent for enhancing the transformation efficiency	Add 0.6 ml of dd-H ₂ O and sterilized with filter, dispense into 50 µl aliquots, stored at -20°C	0.185 g

Introduction

General background of *Yarrowia lipolytica*

Yarrowia lipolytica is a species of non-conventional and GRAS (generally regarded as safe) yeast widely utilized in industrial applications such as organic acid and protein production. As unicellular organism, it has the advantages of *E. coli* and *Saccharomyces cerevisiae* in ease of manipulation and growth capacity. But, it also functions as a higher eukaryotic organization able to perform post-translational processing of complex proteins. As compared to *S. cerevisiae*, *Y. lipolytica* has certain advantages, such as a mainly co-translational secretion pathway (like in mammalian cells), higher secretion capacity and product yield, less hyperglycosylation on products, and simplicities in scaling-up production. These features make *Y. lipolytica* very useful as a protein expression system. Furthermore, the whole genome of *Yarrowia lipolytica* has been sequenced; please check <http://cbl.labri.fr/Genolevures/elt/YALI> for details.

Product Description

YLEX Expression Kit, based on INRA INAPG licensed patent^{*}, provides an easy approach for cloning and expressing a gene of interest in the yeast *Yarrowia lipolytica*. Using this kit, heterologous protein may be expressed intracellularly or secreted from the cell into medium by selecting respectively the supplied expression vector pYLEX1 or pYLSC1.

Using **YLEX Expression Kit**, heterologous protein expression is driven by a strong hybrid promoter (hp4d) carrying four tandem copies of an upstream activator sequence (UAS1B) from pXPR2 and a minimal pLEU2 fragment. To achieve expression in yeast, pYLEX1 containing a cloned gene of interest is linearized by a selected restriction enzyme to produce an expression cassette that can integrate with high efficiency into the *Y. lipolytica* genome by homologous recombination with an integrated pBR platform. A leucine gene (*LEU2*) in pYLEX1 provides for selection of yeast containing an integrated expression cassette by allowing their growth on leucine-free minimal medium. The integrated vector is particularly stable, and targeted monocopy integration allows a direct comparison of the properties of the transformants, which are comparable in terms of locus and copy number.

To achieve secretion of protein from yeast cells, the gene of interest is cloned into pYLSC1, downstream from the *XPR2* pre region (secretion signal from *XPR2* gene), resulting in expression of a secretion signal fusion protein. The *XPR2* pre region directs the fusion protein to be efficiently transported through the yeast secretory pathway. The secretion signal fusion protein undergoes sequential processing by signal peptidase and protease in the endoplasmic reticulum and Golgi complex respectively, resulting in the secretion of the native form of the protein of interest into the culture medium.

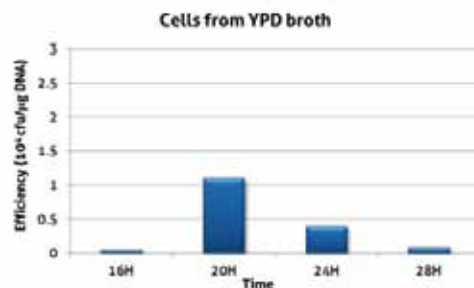
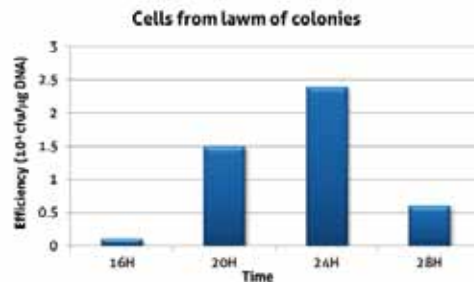
For more information, please read the articles cited in this user's manual.

^{*} INRA (Institut National de la Recherche Agronomique) and INAPG (Institut National Agronomique Paris-Grignon, renamed AgroParisTech since 2007)

Troubleshooting

Low efficiency of transformation

- Test various time lengths (from 10 min to 90 min) of heat shock (at 39°C).
 - Our data indicated that 60-minute heat shock treatment is the optimal condition for Po1g strain and pYLEX1 or pYLSC1 vectors. Results may vary depending on the strain used and the efficiency of plasmid integration into the host chromosome (depending on the length of sequence homology between the plasmid and the host genome).
- The number of yeast cells in the preparation step is critical for the transformation efficiency.
 - Plate approximately 5×10^6 cells onto an YPD plate (Step #1 in "For cells from lawn of colonies").
 - Mix 5×10^7 cells with YLOS cocktail (Step #1 in YLOS One-Step Transformation)
 - Yeast cell density will be preferably determined by counting, using a Mallassez counting chamber according to the specification of the supplier. Alternatively, measurement of optical density at 600 nm (OD_{600}), in a spectrophotometer, can be used (OD readings should be between 0.05 and 0.3 to ensure significance). However, since the relation between cell density and OD is highly variable, depending not only on yeast strain and cultivation conditions, but also on the sensitivity of the apparatus to light scattering, the spectrophotometer should be calibrated by determining independently the cell density in a counting chamber or by performing plating experiments. As a rough guide, a Po1g *Yarrowia* strain culture of 10^7 cells per ml, grown in YPD pH 4 broth, gives an OD_{600} value of approximately 0.3 in a Novaspec II Visible Spectrophotometer.
- Use appropriate amount (5–100 ng) of linearized plasmid DNA. Since circular form will not integrate efficiently, check that linearization of the plasmid with the chosen enzyme (i.e. *NotI* restriction digestion) was effective.
 - A range of 5–40 ng of DNA may show the better efficiency for our Po1g *Yarrowia* strain.
- Carrier DNA should be thawed on ice, avoid thawing it at room temperature.
- The cultivation time of the yeast cells during the preparation step is critical for the transformation efficiency.
 - The figure below shows the transformation efficiencies obtained with yeast cells from lawn of colonies or YPD broth, cultivated at 28°C for 16 to 28 hr. 40 ng of linearized plasmid DNA were used for transformation. The higher transformation efficiency was achieved in both cases with yeast cells cultivated for 20–24 hr (best results were obtained respectively after 24 hr for cells from lawn of colonies, and 20 hr for cells from YPD broth).



Transformants Without Gene Expression

- Test 6 to 12 transformants for the expression of the heterologous gene.
- Among the transformants obtained, only a maximum of 10 to 20% could possibly fail to express the heterologous gene, which could be due to gene conversion (double crossing-over event leading to the replacement by the selection marker gene of the deleted genomic version) or to out-of-site integration (to a locus unfavorable to expression) (Madzak et al., 2004)

Examples

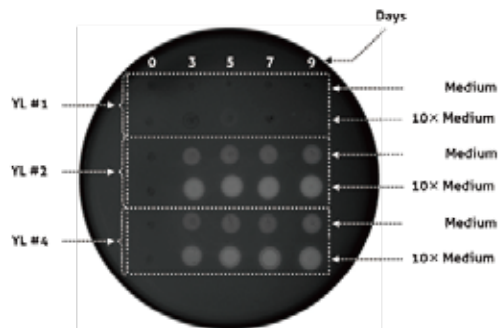
To test whether recombinant α -amylase could be expressed and secreted from yeast by using the YLEX Expression Kit, four different *Yarrowia* transformants were constructed.

Po1g (pYLEX1)	YL #1
Po1g (pYLEX1 + AMY1)	YL #2
Po1g (pYLSC1)	YL #3
Po1g (pYLSC1 + AMY1 Δ)	YL #4

AMY1 : Mouse salivary α -amylase gene

AMY1 Δ : AMY1 without its native secretion signal

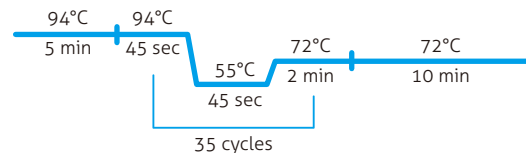
The figure below shows that filtered culture medium from batch culture of both amylase-encoding transformants (YL #2 and #4) could digest starch in solid medium agar, and subsequently produce clear zones. In contrast, medium from the culture of yeast transformed with vector only (YL #1) did not exhibit the same result. This indicates that cloning and expression of respectively α -amylase gene (AMY1) into pYLEX1 and α -amylase gene without its secretion signal peptide (AMY1 Δ) into pYLSC1 have been successful by using the YLEX Expression Kit. In both cases, active α -amylase was efficiently secreted into the culture medium.



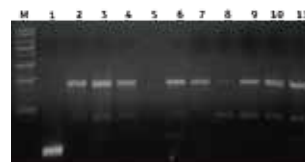
After yeast transformation, plates were incubated at 28°C for 2 days, and 10 transformants from each construction YL #2 and YL #4, were analyzed using PCR on yeast colony. Transformants were resuspended in the PCR mixture described below:

10X PCR buffer	2.5 μ l
dNTPs	0.5 μ l
Primer 6560F	0.5 μ l
Primer 6904R	0.5 μ l
Taq polymerase (5 U/ μ l)	0.5 μ l
==> add water to 25 μ l	

Perform PCR with the following parameters:



The figure below shows the analysis of 10 μ l of PCR product on agarose gel. The size expected for YL #1 or YL #3 (transformation with empty vector) is 340 bp. The size expected for YL #2 or YL #4 (amylase-encoding transformants) is 1.8 kb, indicated by the arrow. The results show that more than 90% of the transformants contain the heterologous gene



Lane M: 1 kb ladder DNA marker
Lane 1: YL #1 [Po1g (pYLEX1)]
Lane 2-11: YL #2 [Po1g (pYLEX1+Amy1)]



Lane M: 1 kb ladder DNA marker
Lane 1: YL #3 [Po1g (pYLSC1)]
Lane 2-11: YL #4 [Po1g (pYLSC1+Amy1 Δ)]

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2. Madzak, C., Treton, B. and Blanchin-Roland, S. (2000) Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J. Mol. Microbiol. Biotechnol.* 2:207-216.
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Other important related articles

1. Madzak, C., Blanchin-Roland, S., Cordero Otero, R.R., and Gaillardin C. (1999) Functional analysis of upstream regulating regions from the *Yarrowia lipolytica* XPR2 promoter. *Microbiology* 145:75-87.
2. Nicaud, J.-M., Madzak, C., van den Broek, P., Gysler, C., Duboc, P., Niederberger, P., and Gaillardin, C. (2002) Protein expression and secretion in the yeast *Yarrowia lipolytica*. *FEMS Yeast Res.* 2:371-379.
3. Jolival, C., Madzak, C., Brault, A., Caminade, E., Malosse, C., and Moug, C. (2005) Expression of laccase IIIb from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica* for environmental applications. *Applied Microbiol. Biotechnol.* 66(4):450-456.
4. Madzak, C., Otterbein, L., Chamkha, M., Moukha, S., Asther, M., Gaillardin, C. and Beckerich, J.-M. (2005) Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. *FEMS Yeast Res.* 5(6-7):635-646.
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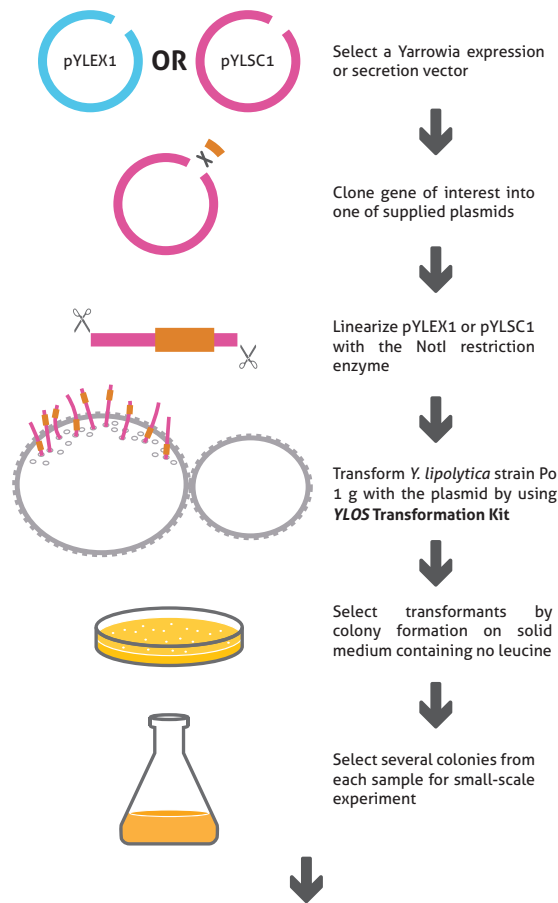
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Experimental Process



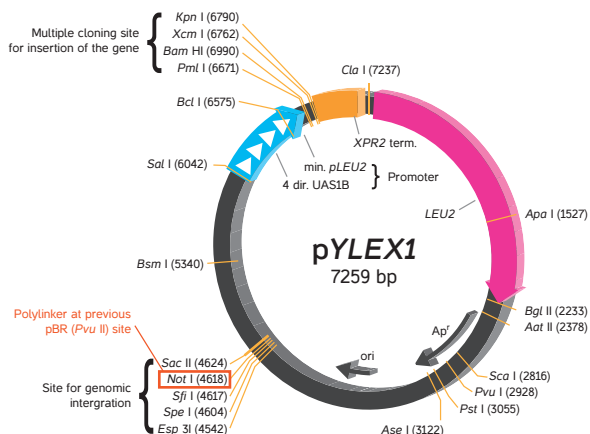
Analyze the protein of interest

Yarrowia Vectors

Two vectors (pYLEX1 and pYLSC1) are included in this kit, and they can be used for either intracellular expression or secretion of proteins of interest in *Y. lipolytica*. Generally speaking, if the target protein is cytosolic and non-glycosylated, the pYLEX1 vector is a better choice. If the protein of your interest is normally glycosylated or secreted, you may wish to choose the pYLSC1 vector. To secrete the gene of interest, we recommend that you try both pYLEX1 with native secretion signal (if applicable) and pYLSC1 (containing XPR2 pre region) to express and secrete the protein. The following sections describe various factors that affect how genes should be cloned into pYLEX1 or pYLSC1 to achieve the desired method of expression.

Selecting a Yarrowia Expression Vector (pYLEX1)

The map of pYLEX1



The pYLEX1 expression vector (7259 bp) contains the strong hybrid promoter (hp4d) carrying four tandem copies of upstream activator sequences (UAS1B) fragment from pXPR2 and a minimal pLEU2 fragment. The multiple cloning site and the XPR2 transcription terminator lie immediately downstream from hp4d promoter. They are followed by a leucine selection marker gene (LEU2). The vector can be linearized by digestion with NotI (in the pBR region) to create a linear DNA fragment capable of inserting into the *Y. lipolytica* genome, at the pBR docking platform of Po1g strain.

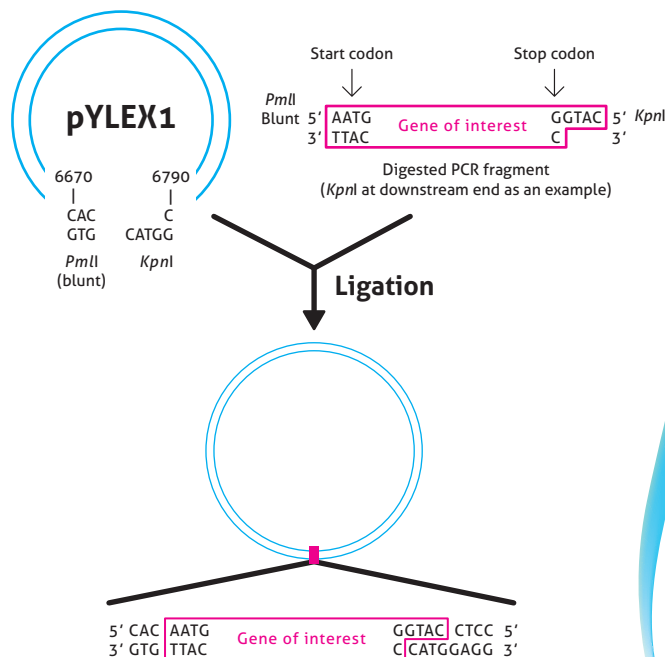
Multiple cloning site in pYLEX1

pYLEX1 vector contains the following restriction sites for inserting the gene of interest.

6651	ATACAACCAC	ACACATCCAC	<u>Pml</u> GTGGGAACCC	GAAACTAAGG	<u>BamHI</u> ATCCAACCTAC
6701	GGAACCTGTG	TTGATGTCTT	TGCCCCCGGC	TCCGATATCA	TCTCTGCCTC
6751	<u>XcmI</u> TTACCAGTCC	GACTCTGGTA	CTTGGGTCTA	<u>Acc65I</u> CTCCGGTACC	TCCATGGCCT

Cloning of a heterologous gene into pYLEX1

Digest vector with *Pml* and a selected downstream enzyme (*KpnI* as an example)



Brief outlines for cloning a PCR fragment into pYLEX1:

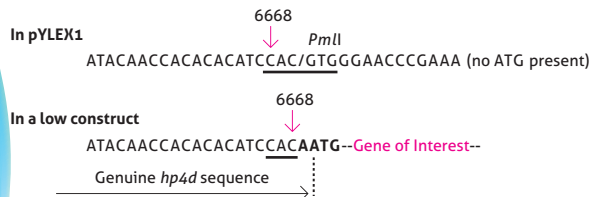
1. Prepare a PCR product of the gene of interest.
The PCR fragment must have a blunt end at its upstream end with AATG sequence, and a stop codon followed by a cut site (*Bam*HI, *Xcm*I or *Kpn*l) at its downstream end.
2. Use the selected restriction enzyme (*Bam*HI, *Xcm*I or *Kpn*l) to digest the PCR fragment 3' downstream end.
3. Purify the digested PCR fragment by using a commercially available gel extraction kit.
4. Use the same selected enzyme (from step 2) and *Pml*l restriction enzyme to digest the vector pYLEX1.
5. DNA ligation. Mix the modified PCR fragment and the linear vector DNA with T4 DNA ligase.
6. Transformation. Add the ligation mixture to competent *E. coli* cells.
7. Prepare miniprep DNA from transformants. Digest each with an appropriate restriction endonuclease to determine the presence of a cloned insert and vector size.
8. ★ Digest the cloning plasmid with *Not*I restriction enzyme for yeast transformation. Alternatively, if *Not*I is present in the gene of interest, other choices are possible (see "Sites for genomic integration" in the map of pYLEX1).

To construct the vector pYLEX1, the original ATG of the hybrid promoter (*hp4d*) in the parent vector was replaced by a *Pml*l blunt cloning site. The *Pml*l site can be used to obtain a perfect fusion between the hybrid promoter and the heterologous gene.

Native promoter sequence: 5' ATCCACAATGGAACCC

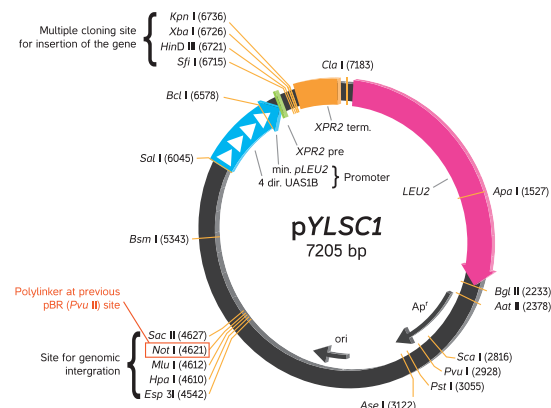
Modified sequence in pYLEX1: 5' ATCCA/GTGGGAACCC
PmlI blunt site

Therefore, the end of the promoter (the sequence AATG) must be reconstituted at the same spot in order to express the heterologous gene properly (as in the graph shown below). The gene of interest (or a PCR fragment) with AATG sequence must be inserted into the *Pml*l blunt site of pYLEX1 using its blunt upstream end. The downstream end of the gene can be ligated to *Bam*HI or *Kpn*l unique sites.



Selecting a *Yarrowia* Secretion Vector (pYLSC1)

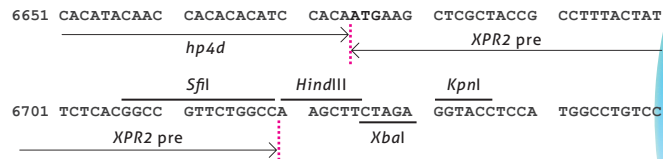
The map of pYLSC1



The pYLSC1 secretion vector (7205 bp) contains the hybrid promoter (*hp4d*) and a secretion signal (*XPR2* pre region). The multiple cloning site and the p*XPR2* transcription terminator lie immediately downstream of 3' end of *XPR2* pre region. They are followed by a leucine selection marker gene (*LEU2*). The vector can be linearized by digestion with *Not*I (in the pBR region) to create a linear DNA fragment capable of inserting into the *Y. lipolytica* genome, at the pBR docking platform of Po1g strain.

Multiple cloning site in the pYLSC1 vector

pYLSC1 contains the following restriction sites for inserting the gene of interest.



1. Prepare a PCR product of the gene of interest.
The PCR fragment must contain the sequence of the *Sfi* site from *XPR2* pre region at its upstream end, and a stop codon followed by a cut site (*HindIII*, *XbaI* or *KpnI*) at its downstream end.
2. Use *Sfi* and the selected restriction enzyme (*HindIII*, *XbaI* or *KpnI*) to digest both the PCR fragment and the vector pYLSC1.

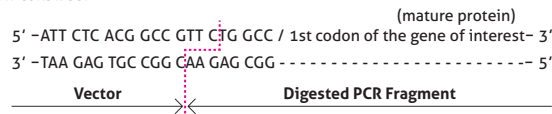
3. Purify the digested PCR fragment by using a commercially available gel extraction kit.
4. DNA ligation. Mix the modified PCR fragment and the linear vector DNA with T4 DNA ligase.
5. Transformation. Add the ligation mixture to competent *E. coli* cells.
6. Prepare miniprep DNA from transformants. Digest each with an appropriate restriction endonuclease to determine the presence of a cloned insert and vector size.
7. ★ Digest the cloning plasmid with *NotI* restriction enzyme for yeast transformation. Alternatively, if *NotI* is present in the gene of interest, other choices are possible (see "Sites for genomic integration" in the map of pYSLC1).

The secretion vector (pYLS_{C1}) carries a transcriptional fusion of the hybrid promoter (hp4d) to the *KPR2* pre region (secretion signal), allowing the secretion of the expressed heterologous protein. In this vector, the end of the *KPR2* pre region has been modified, while respecting amino acid coding, to create a *Sfi*I restriction enzyme site, used to generate a translational fusion between the *KPR2* pre region and the gene. The gene of interest (or a PCR fragment) must be ligated to *Sfi*I site and reconstitute the end of the pre sequence, up to the cleavage site. The downstream end of the gene of interest can be ligated to one of three available restriction enzyme sites (*Hind*III, *Xba*I and *Kpn*I).

In pYLSC1



In a new construct



YLOS One-Step Transformation

Introduction

YLOS One-Step Transformation system provides a simple way to transform *Yarrowia* cells cultured in either solid agar or liquid broth. They were designed for various strains of *Yarrowia lipolytica* and vectors, including Po1g strain and pYLEX1 or pYLSC1 vectors in this kit. Transformation efficiency may vary with each strain and vector used.

Before Beginning

Prepare the appropriate selection media, and pour the required number of agar plates.

We suggest the use of YNB medium (N_{3000}):

- 20 g/L glucose
- 6.7 g/L yeast nitrogen base w/o amino acids
(or 1.7 g/L yeast nitrogen base w/o amino acids and ammonium sulfate
+ 5 g/L ammonium sulfate)
- 15 g/L agar

Note:

1. Fully dried plates always give higher efficiency in YLOS transformation system.
2. Suggestion: uncover plates in a laminar flow for approximate 1 hour until the surface is dry.

Prepare the appropriate amount of either YPD agar plates, or YPD pH 4 liquid broth.

- 20 g/L glucose
- 10 g/L yeast extract
- 20 g/L bacto peptone

For plates:

- Add 15 g/L agar

For pH 4 broth:

- Add 50 mM Citrate Buffer pH 4
(add from sterile 0.5 M stock following broth autoclaving)

YLOS Cocktail (Prepare just prior to transformation)

For each transformation:

1. Add 95 μ l of YLOS buffer in a 1.5 ml sterile microcentrifuge tube.
2. Add 5 μ l of DTT solution (stock at -20°C , thaw on ice or quick thaw at room temperature in a water bath).
3. Add 2.5 μ l of carrier DNA (stock at -20°C , thaw on ice).
4. Add appropriate amount (5–100 ng) of linearized plasmid DNA (in a maximal volume of 5 μ l).

Preparation of Yeast Cells:

For cells from lawn of colonies:

1. Pick up a single colony of *Yarrowia*, and streak or spread completely on a YPD plate.
Note: For a better result, resuspend the colony in 0.5 ml of ddH₂O, then plate approximately 5×10^6 cells onto an YPD plate)
2. Incubate the plate at 28°C for 16–24 hr.

3. Scrape the lawn of cells from agar surface, and wash cells with 1 ml of sterile ddH₂O twice (resuspend softly, centrifuge at 3000 rpm at room temperature for 5 min, discard supernatant).

Note: For a better result, cells should be collected at 5×10^7 per tube, after the second washing.

4. Save the pellets and proceed to transformation.

For cells from YPD broth:

1. Inoculate a single colony of your *Yarrowia* strain in a 250-ml flask containing 10 ml of YPD pH 4 liquid broth. Grow at 28°C until saturated (20–22 hr) in a shaking incubator (250–300 rpm).

2. Harvest the cells at a cell density of $\sim 5 \times 10^8/\text{ml}$.

3. Centrifuge the cells at 3000 rpm at room temperature for 5 min. Discard the supernatant.

4. Wash the cell pellet with sterile dd-H₂O twice to deplete all residues of YPD broth.

5. Resuspend softly the cells with 1 ml of 0.1 M LiOAc (pH=6.0).

6. Transfer 0.1 ml of the cells to a new sterile microcentrifuge tube.

Note: This corresponds to 5×10^7 cells per tube.

7. Centrifuge the cells at 3000 rpm at room temperature for 5 min, and discard the supernatant.

8. Save the pellets and proceed to transformation.

One-Step Transformation

For each transformation:

1. Resuspend the cells with freshly prepared YLOS cocktail (corresponds to 5×10^7 cells/tube).

2. Incubate the tubes at 39°C for 60 min.

3. Plate the entire "transformation cocktail" on appropriate "dried" selection plates (YNB).

Note: It is highly suggested to spread the transformation cocktail by using preferably a sterile glass rod, or alternatively sterile glass beads (4mm).

4. Incubate the plates at 28°C for 2–4 days.

